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Pulmonary Damage by *Vibrio vulnificus* Cytolysin

JIN-WOO PARK,[1] SEONG-NAM MA,[1] EUN-SUP SONG,[1]† CHANG-HO SONG,[1] MEE-REE CHAE,[1] BYUNG-HYUN PARK,[1] HYE-WON RHO,[1] SEOK-DON PARK,[2] AND HYUNG-RHO KIM[1]*

Department of Biochemistry and Anatomy, School of Medicine and Institute for Medical Sciences, Chonbuk National University, Chonju, 560-182,[1] and Department of Dermatology, Wonkwang University, School of Medicine, Iksan, 570-749,[2] Republic of Korea

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***Vibrio vulnificus* is an estuarine bacterium that causes septicemia and serious wound infection. Cytolysin produced by *V. vulnificus* has been incriminated as one of the important virulence determinants of bacterial infection. Cytolysin (8 hemolytic units) given intravenously to mice via their tail veins caused severe hemoconcentration and lethality. Cytolysin treatment greatly increased pulmonary wet weight and vascular permeability as measured by ^{125}I-labeled albumin leakage without affecting those factors of other organs significantly. Blood neutrophils were markedly decreased in number after cytolysin injection, with a concomitant increase in the level of pulmonary myeloperoxidase activity, indicating that cytolysin-induced neutropenia might be due to pulmonary sequestration of neutrophils. By microscopic examination, severe perivascular edema and neutrophil infiltration were evident in lung tissues. These results suggest that increased vascular permeability and neutrophil sequestration in the lungs are important factors in lethal activity by cytolysin.**

Vibrio vulnificus is an estuarine bacterium that causes septicemia and serious wound infections in persons who are immunocompromised or have underlying diseases, such as liver cirrhosis or hemochromatosis (7, 25). To establish cytolysin as one of a number of possible virulence determinants, a previous study by Kreger and Lockwood (17) demonstrated that the cytolysin present in a culture medium of *V. vulnificus* showed a cytotoxicity for mammalian cells in culture and acted as a vascular permeability factor.

V. vulnificus cytolysin, produced by most pathogenic strains, is a water-soluble polypeptide with a M_r of 51,000 (14, 22). *V. vulnificus* cytolysin is extremely toxic, and even a submicrogram amount of cytolysin is fatal to mice when injected intravenously. We already reported that hemolysis caused by *V. vulnificus* cytolysin is colloid-osmotic in nature and that cytolysins, after binding to membranes, were oligomerized to form Ca^{2+} ion-impermeable small pores on cell membranes (15, 24). But the recognition that cytolysin generates transmembrane pores does not in itself explain its pathogenetic role in bacterial infections. Cell surface factors, although poorly understood, may influence the binding and oligomerization of toxins on target cells, and various cell types may differ widely and unpredictably in their susceptibilities toward a given toxin (6). Little is known about the target cell of *V. vulnificus* cytolysin in the body and the relationship between its lethal activity and the cytotoxic mechanism involving pore formation.

Cytolysin injected subcutaneously into mice caused severe structural alteration of skin, and the tissue damage induced by cytolysin was very similar to that shown in *V. vulnificus* wound infections (12). In this report, we have studied the systemic effects of *V. vulnificus* cytolysin injected via the tail veins of mice and demonstrated that the lungs are the major target organ and that neutrophils might be involved in the pulmonary damage and lethality of cytolysin.

* Corresponding author. Mailing address: Department of Biochemistry, Chonbuk National University Medical School, Chonju, 560-182, Republic of Korea. Phone: 82-652-70-3081. Fax: 82-652-74-9833.

† Present address: Department of Dermatology, Presbyterian Medical Center, Chonju, 560-750, Republic of Korea.

A virulent strain of *V. vulnificus*, E4125, was kindly supplied by M. H. Kothary (Department of Microbiology, Virulence Assessment Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, D.C.). The strain was cultured in heart infusion diffusate broth (Gibco) at 37°C for 4 h as described by Kreger et al. (18), and cytolysin was homogeneously purified from the culture supernatant by ammonium sulfate fractionation, calcium phosphate gel adsorption, quaternary methylamine anion-exchange chromatography, and octyl-Sepharose CL-4B chromatography as described by Kim et al. (14).

The hemolytic activity of cytolysin against mouse erythrocytes was determined by the method of Bernheimer and Schwartz (3). The cytolysin was diluted with phosphate-buffered saline (PBS) (67 mM Na_2HPO_4, 77 mM NaCl [pH 7.4]) containing 1 mg of bovine serum albumin (BSA) per ml. One milliliter of cytolysin was mixed with the same volume of 0.7% mouse erythrocyte suspensions in PBS-BSA. After incubation at 37°C for 30 min and brief centrifugation, A_{545} of hemoglobin in the supernatant was measured. One hemolytic unit (HU) is defined as that amount which liberates half of the hemoglobin in the erythrocyte suspensions.

Cytolysin diluted in 0.1 ml of PBS-BSA at doses of 1, 2, 4, 8, and 16 HU was injected intravenously via the tail veins of adult male BALB/c mice (25 to 30 g each) in groups of five. The control group was injected with PBS-BSA. About half of the animals were killed at a dose of 4 HU in 24 h, and all the mice were killed in about 6 h at a dose of 8 HU (100 ng). Our findings were similar to those of Gray and Kreger (11) in that the 50% lethal dose of purified *V. vulnificus* cytolysin for mice was 6 HU when it was administered intravenously. Many cytolysins, such as *Escherichia coli* hemolysin or staphylococcal alpha-toxin, usually require higher doses for lethal activity in in vivo experiments because of the inactivation by plasma components (4, 5). In contrast, *V. vulnificus* cytolysin was stable in mouse plasma (data not shown). At various times from 0 to 4 h after the intravenous injection of cytolysin at a dose of 8 HU, changes in the hematocrits were determined (Fig. 1). Hematocrits began to increase after 30 min, reaching 65% in 4 h. These results suggest that cytolysin caused an increase in the

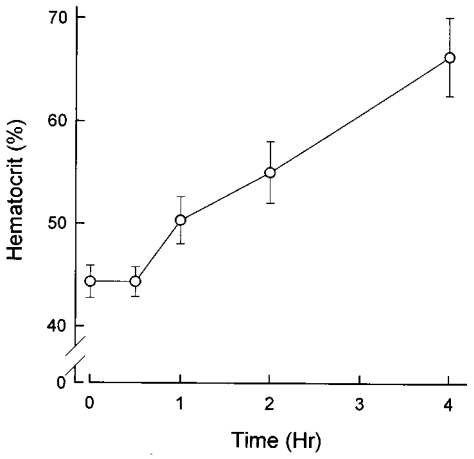

FIG. 1. Effects of cytolysin on hematocrits of mice. Cytolysin in a dose of 8 HU was injected into mice via their tail veins. At various times, blood hematocrits were determined. Each point represents the mean ± the standard error from five mice.

level of extravasation of fluid from the blood vessels and consequent hypovolemic shock.

To investigate the target organ of cytolysin, tissue injury was assessed after the injection of cytolysin at a dose of 8 HU (Table 1). The amount of tissue injury was estimated by the increase of both wet weight and vascular permeability estimated from the leakage of ^{125}I-BSA (29, 31). After concomitant injection of cytolysin and 0.1 μCi of ^{125}I-BSA, tissues were dissected at 4 h, washed in cold saline, and weighed, and their levels of radioactivity were determined. Vascular permeability was expressed as the tissue-to-blood ratio of ^{125}I-BSA. The ^{125}I-BSA was prepared by the standard method with Iodo-Gen (1,3,4,6-tetrachloro-3 α,6 α-diphenylglycouril; Sigma) (8). Among the tissues studied, a significant increase in wet weight was observed only in lung tissues at 4 h after injection, without significant changes in the wet weights of other tissues, including those of the liver, kidney, spleen, heart, and brain. The permeability indexes calculated from the leakage of the ^{125}I-labeled BSA showed a great increase above normal levels only for the lung tissues.

For the microscopic examination, excised tissues were fixed in 10% neutral-buffered formalin (0.1 M phosphate, pH 7.4). Samples were washed, dehydrated with graded alcohols, and embedded in paraffin. Six-micrometer sections were stained by hematoxylin and eosin. Under the microscope, severe perivascular edema and congestion were evident in the lung tissues (Fig. 2B). No significant morphologic changes were observed in other organs during the 4 h after cytolysin injection.

These results indicate that increased pulmonary vascular permeability and resulting hypovolemic shock are the major causes of death in mice and that the lungs are the major target organ of cytolysin in the body.

Severe leukopenia was found at 1 h after cytolysin injection of 8 HU and persisted until 4 h had passed (Fig. 3). Leukocytes in blood samples were counted with a hemocytometer. Most of the removed leukocytes were neutrophils (data not shown). The level of pulmonary myeloperoxidase activity began to increase with a concomitant increase of lung tissue wet weight (Fig. 3). Myeloperoxidase is a constituent enzyme of azurophilic neutrophil granules constituting 5% of neutrophil dry weights (16, 28) and has been used as an indicator of neutrophil accumulation in lung tissues (2, 9).

Myeloperoxidase activity in lung tissue was measured by the method of Anderson et al. (2) with slight modification. Lung tissues were washed with saline, homogenized for 30 s in 2 ml of 20 mM potassium phosphate buffer (pH 7.4) with a Polytron homogenizer (Kinematica), and centrifuged at 15,000 × *g* for 30 min at 4°C (Sorvall; DuPont). After being washed again, the pellet was resuspended in 2 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 5 mg of hexadecyltrimethyl ammonium bromide per ml. After sonication for 90 s and centrifugation at 15,000 × *g* for 15 min, the supernatant was used to assay myeloperoxidase activity.

One hundred microliters of supernatant was added to 1.4 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg of *O*-dianisidine and 0.005% H_2O_2 per ml, and the A_{460} was measured for 3 min (9). When necessary, samples were diluted in 50 mM potassium phosphate buffer (pH 6.0) containing 5 mg of hexadecyltrimethyl ammonium bromide per ml to maintain the linearity of the time-absorbance plot. One unit of myeloperoxidase activity is the amount of enzyme that will reduce 1 μmol of peroxide per min.

Cytolysin-induced neutropenia and the concomitant rise in the level of pulmonary myeloperoxidase activity indicate that pulmonary sequestration of neutrophils might be involved in the pulmonary damage and lethality of cytolysin. Microscopic findings of increased pulmonary neutrophil infiltration also supported these results (Fig. 2C).

Multiple-organ failure almost invariably begins with pulmonary dysfunction (20). The weight of evidence indicates that multiple-organ failure by various causes, including bacterial endotoxins, is mediated largely by neutrophils (10, 21, 27, 30–32). The source of these destructive cells is thought to be marginating neutrophils that remain closely associated with the endothelia of the small vessels from the mainstream blood flow (2). They ultimately adhere to endothelia, migrate across basement membranes, and degranulate in tissue interstitia to cause tissue injury and dysfunction (2, 19, 33). In the lungs, with their extensive low-pressure microcirculation, large numbers of marginating neutrophils reside primarily in capillaries (1, 26). Our results indicate that increased pulmonary vascular permeability and neutrophil sequestration are important in the lethal activity of cytolysin injected intravenously. It is tempting to speculate that the marginating neutrophils, which predispose the lungs to destruction by virtue of their large size, their predilection for lung tissues, and their tremendous destructive po-

TABLE 1. Effects of cytolysin on wet weights and vascular permeabilities of mouse tissues

Tissue	Mean ± SE of data measuring[a]:			
	Wet weight (mg) of:		Permeability index[b] of:	
	Control mice	Cytolysin-treated mice	Control mice	Cytolysin-treated mice
Lung	206 ± 15	349 ± 19[c]	0.35 ± 0.04	1.64 ± 0.31[d]
Liver	2,001 ± 12	1,989 ± 32	0.21 ± 0.07	0.16 ± 0.01
Kidney	265 ± 20	276 ± 31	0.22 ± 0.05	0.22 ± 0.05
Spleen	115 ± 15	129 ± 9	0.13 ± 0.02	0.15 ± 0.03
Heart	166 ± 25	172 ± 12	0.18 ± 0.01	0.19 ± 0.05
Brain	380 ± 32	337 ± 13	0.05 ± 0.02	0.05 ± 0.02

[a] Data are expressed as means ± standard errors of results obtained from five mice of each group, and comparisons between groups were performed by the unpaired Student's *t* test.

[b] The permeability index is expressed as a tissue-to-blood ratio of a ^{125}I-BSA radioactivity level.

[c] $P < 0.01$.

[d] $P < 0.05$.

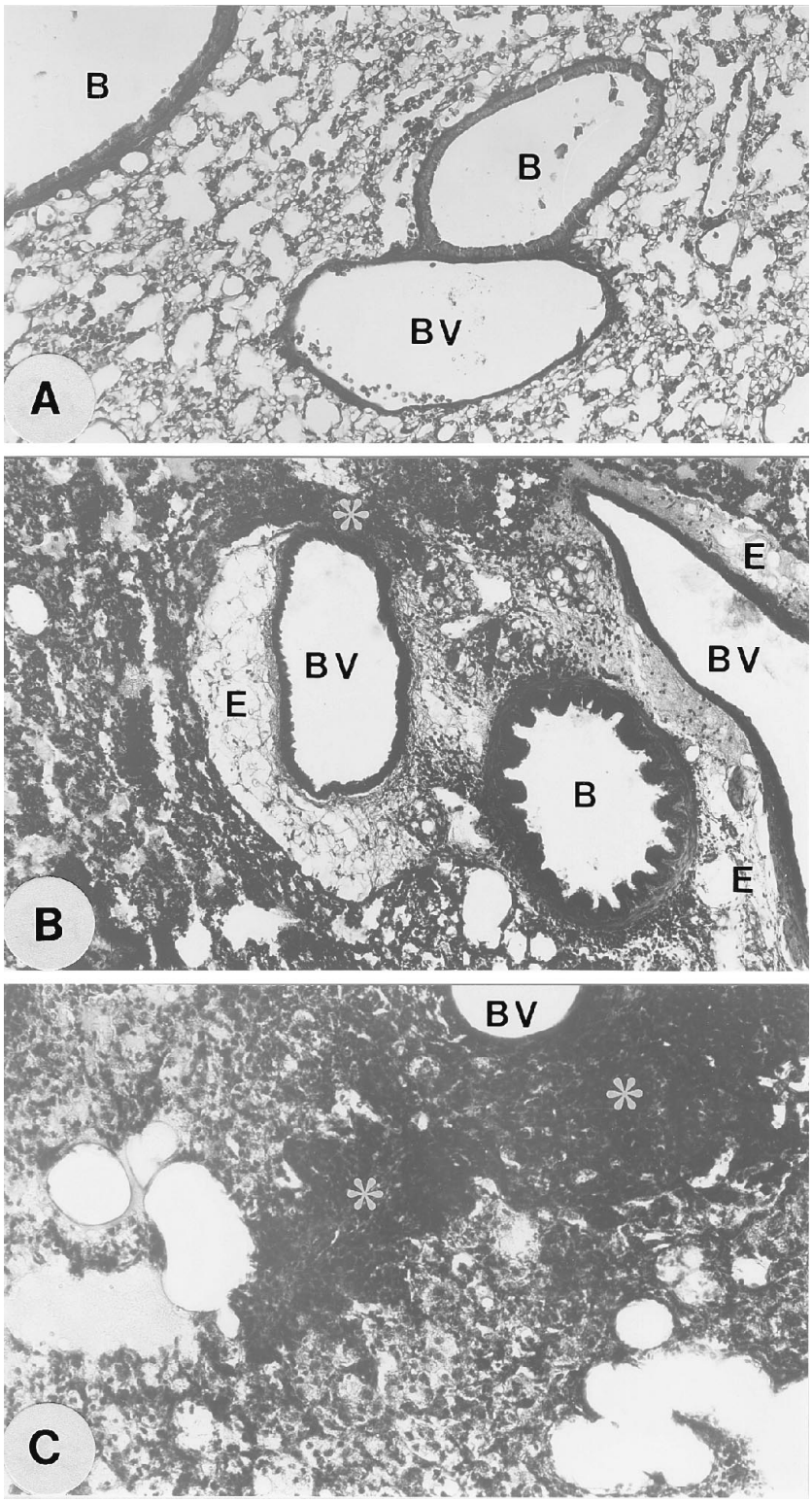

FIG. 2. Effects of cytolysin on the morphologic change of lung tissues. (A) Light micrograph of mouse lung tissue at 4 h after intravenous injection of PBS-BSA. A blood vessel is adjacent to a bronchiole. The alveoli are shown as small polyhedral chambers. Magnification, ×200. (B and C) Light micrographs of mouse lung tissues at 4 h after intravenous injection of 8 HU of cytolysin. (B) The perivascular edema and infiltrating leukocytes (asterisks) are shown. Magnification, ×100. (C) Note the widespread interstitial leukocytes that have infiltrated the perivascular area. Many of the alveoli are filled with edema fluid and leukocytes. Magnification, ×200. Abbreviations: BV, blood vessel; B, bronchiole; E, perivascular edema.

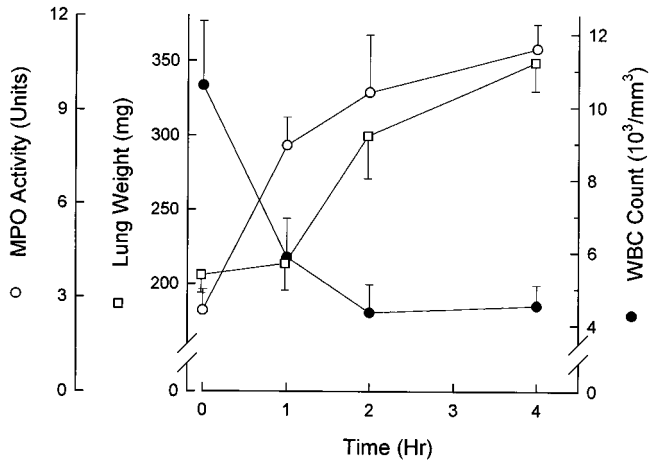

FIG. 3. Effects of cytolysin on leukocytes, pulmonary wet weights, and myeloperoxidase activity. Cytolysin in a dose of 8 HU was injected into mice via their tail veins. At various times, the numbers of leukocytes (WBC) in blood samples were determined with a hemocytometer and pulmonary wet weights and levels of myeloperoxidase (MPO) activity were determined as described in Materials and Methods. Each point represents the mean ± the standard error from five mice.

tential, play the central role in cytolysin-induced lethality for mice.

The pathogenetic roles of cytolysin in *V. vulnificus* infection are controversial (23, 34), but cytolysin is very powerful and is still one of the most likely candidates for the pathogenesis of disease (22). Gray and Kreger (13) detected cytolysin in an extract from a skin lesion and in sera from mice showing local and systemic *V. vulnificus* disease. Our study used a lethal dose of purified cytolysin, and it was injected in bolus via tail veins. Massive pulmonary damage could be explained in terms of the first capillary beds exposed to cytolysin injected intravenously. Further study is required to coordinate our results with *V. vulnificus* infection and to find the original reason cytolysin causes sequestration of neutrophils and organ damage.

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REFERENCES

1. **Albelda, S. M., M. J. Karnovsky, and A. P. Fishman.** 1987. Perspectives in endothelial cell biology. J. Appl. Physiol. **62:**1345–1348.
2. **Anderson, B. O., J. M. Brown, P. F. Shanley, D. D. Bensard, and A. H. Harken.** 1991. Marginating neutrophils are reversibly adherent to normal lung endothelium. Surgery **109:**51–61.
3. **Bernheimer, A. W., and L. L. Schwartz.** 1963. Isolation and composition of staphylococcal alpha-toxin. J. Gen. Microbiol. **30:**455–468.
4. **Bhakdi, S., S. Greulich, M. Muhly, B. Eberspacher, H. Becker, A. Thiele, and F. Hugo.** 1989. Potent leukocydal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. J. Exp. Med. **169:**737–754.
5. **Bhakdi, S., M. Muhly, U. Mannhardt, F. Hugo, K. Klapettek, C. Mueller-Eckhardt, and L. Roka.** 1988. Staphylococcal α toxin promotes blood coagulation via attack on human platelets. J. Exp. Med. **168:**527–542.
6. **Bhakdi, S., and J. Tranum-Jensen.** 1988. Damage to cell membranes by pore-forming bacterial cytolysins. Prog. Allergy **40:**1–43.
7. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine Vibrio: clinical characteristics and epidemiology. N. Engl. J. Med. **300:**1–5.
8. **Fraker, P. J., and J. C. Speck, Jr.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3 α,6 α-diphenylglycouril. Biochem. Biophys. Res. Commun. **80:**849–857.
9. **Goldblum, S. E., K. M. Wu, and M. Jay.** 1985. Lung myeloperoxidase as a measure of pulmonary leukostasis in rabbits. J. Appl. Physiol. **59:**1978–1985.
10. **Goris, R. J., T. P. LeBoekhorst, J. K. Nuytinck, and J. S. Gimbrere.** 1985. Multiple-organ failure: generalized autodestructive inflammation? Arch. Surg. **120:**1109–1115.
11. **Gray, L. D., and A. S. Kreger.** 1985. Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. Infect. Immun. **48:**62–72.
12. **Gray, L. D., and A. S. Kreger.** 1987. Mouse skin damage caused by cytolysin from *Vibrio vulnificus* and by *V. vulnificus* infection. J. Infect. Dis. **155:**236–241.
13. **Gray, L. D., and A. S. Kreger.** 1989. Detection of *Vibrio vulnificus* cytolysin in *V. vulnificus*-infected mice. Toxicon **27:**439–464.
14. **Kim, H. R., S. D. Park, J. W. Park, M. H. Jeong, J. S. Kim, and B. H. Park.** 1992. Purification and characterization of cytolysin produced by *Vibrio vulnificus*. Korean J. Biochem. **24:**7–11.
15. **Kim, H. R., H. W. Rho, M. H. Jeong, J. W. Park, J. S. Kim, B. H. Park, U. H. Kim, and S. D. Park.** 1993. Hemolytic mechanism of cytolysin produced from *V. vulnificus*. Life Sci. **53:**571–577.
16. **Kinkade, J. M., Jr., S. O. Pember, K. C. Barnes, R. Shapira, J. K. Spitznagel, and L. E. Martin.** 1983. Differential distribution of distinct forms of myeloperoxidase in different azurophilic granule subpopulations from human neutrophils. Biochem. Biophys. Res. Commun. **114:**296–303.
17. **Kreger, A., and D. Lockwood.** 1981. Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. Infect. Immun. **33:**583–590.
18. **Kreger, A. S., M. H. Kothary, and L. D. Gray.** 1988. Cytolytic toxins of *Vibrio vulnificus* and *Vibrio damsela*. Methods Enzymol. **165:**176–189.
19. **McDonald, R. J., E. M. Berger, and J. E. Repine.** 1987. Neutrophil-derived oxygen metabolites stimulate thromboxane release, pulmonary artery pressure increases, and weight gains in isolated perfused rat lungs. Am. Rev. Respir. Dis. **135:**957–959.
20. **McMenamy, R. H., R. Birkhahn, G. Oswald, R. Reed, C. Rumph, N. Vaidyanath, L. Yu, F. B. Cerra, R. Sorkness, and J. R. Border.** 1981. Multiple systems organ failure: I. The basal state. J. Trauma **21:**99–114.
21. **Meyrick, B., and K. L. Brigham.** 1983. Acute effects of *Escherichia coli* endotoxin on the pulmonary microcirculation of anesthetized sheep: structure:function relationships. Lab. Invest. **48:**458–470.
22. **Miyoshi, S., E. G. Oh, K. Hirata, and S. Shinoda.** 1993. Exocellular toxic factors produced by *Vibrio vulnificus*. J. Toxicol. Toxin Rev. **12:**253–288.
23. **Oliver, J. D., J. E. Wear, M. B. Thomas, M. Warner, and K. Linder.** 1986. Production of extracellular enzymes and cytotoxicity by *Vibrio vulnificus*. Diagn. Microbiol. Infect. Dis. **5:**99–111.
24. **Park, J. W., T. A. Jahng, H. W. Rho, B. H. Park, N. H. Kim, and H. R. Kim.** 1994. Inhibitory mechanism of Ca^{2+} on the hemolysis caused by *Vibrio vulnificus* cytolysin. Biochim. Biophys. Acta **1194:**166–170.
25. **Park, S. D., H. S. Shon, and N. J. Joh.** 1991. *Vibrio vulnificus* septicemia in Korea: clinical and epidemiologic findings in seventy patients. J. Am. Acad. Dermatol. **24:**397–403.
26. **Perlo, S., A. A. Jalowayski, C. M. Durand, and J. B. West.** 1975. Distribution of red and white blood cells in alveolar walls. J. Appl. Physiol. **38:**117–124.
27. **Repine, J. E., C. M. Bowman, and R. M. Tate.** 1982. Neutrophils and lung edema. Chest **81:**47S–51S.
28. **Schultz, J., and K. Kaminker.** 1962. Myeloperoxidase of the leukocyte of normal human blood. I. Content and localization. Arch. Biochem. Biophys. **96:**465–467.
29. **Seekamp, A., M. S. Mulligan, G. O. Till, C. W. Smith, M. Miyasaka, T. Tamatani, R. F. Todd III, and P. A. Ward.** 1993. Role of β 2 integrins and ICAM-1 in lung injury following ischemia-reperfusion of rat hind limbs. Am. J. Pathol. **143:**464–472.
30. **Smedly, L. A., M. G. Tonnesen, R. A. Sandhaus, C. Haslett, L. A. Guthrie, R. B. Johnston, Jr., P. M. Henson, and G. S. Worthen.** 1986. Neutrophil-mediated injury to endothelial cells: enhancement by endotoxin and essential role of neutrophil elastase. J. Clin. Invest. **77:**1233–1243.
31. **Till, G. O., K. J. Johnson, R. Kunkel, and P. A. Ward.** 1982. Intravascular activation of complement and acute lung injury: dependency on neutrophils and toxic oxygen metabolites. J. Clin. Invest. **69:**1126–1135.
32. **Weiss, S. J.** 1989. Tissue destruction by neutrophils. N. Engl. J. Med. **320:** 365–376.
33. **Worthen, G. S., C. Haslett, A. J. Rees, R. S. Gumbay, J. E. Henson, and P. M. Henson.** 1987. Neutrophil-mediated pulmonary vascular injury: synergistic effect of trace amounts of lipopolysaccharide and neutrophil stimuli on vascular permeability and neutrophil sequestration in the lung. Am. Rev. Respir. Dis. **136:**19–28.
34. **Wright, A. C., and J. G. Morris.** 1991. The extracellular cytolysin of *Vibrio vulnificus*: inactivation and relationship to virulence in mice. Infect. Immun. **59:**192–197.

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